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Osmotic regulation of the Na^+/*myo*-inositol cotransporter and postinduction normalization

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Osmotic regulation of the Na^+/*myo*-inositol cotransporter and postinduction normalization.

Background. In renal cells, hyperosmolarity has been shown to induce the accumulation of *myo*-inositol, via the Na^+/*myo*-inositol cotransporter (SMIT). Previously we showed that SMIT mRNA in the kidney is localized in the medullary thick ascending limb of Henle (TALH). Here we used renal cells derived from the rabbit outer medullary TALH to examine the regulation of *myo*-inositol transport by hyperosmolarity. In addition, using both cultured renal and endothelial cells, we examined the normalization of SMIT activity and mRNA levels following induction by hyperosmolarity.

Methods. TALH cells were exposed to isotonic or hyperosmotic medium, and then SMIT mRNA levels and *myo*-inositol accumulation were determined. To examine postinduction normalization, cultured endothelial and renal cells were first exposed to hyperosmotic medium and then to isotonic medium containing actinomycin D or cycloheximide. Afterwards, SMIT mRNA levels and *myo*-inositol accumulation were determined.

Results. Hyperosmolarity increased SMIT mRNA levels and *myo*-inositol accumulation in TALH cells. The hyperosmolarity-induced increase in *myo*-inositol uptake by TALH cells was characterized by an increase in the V_{max} for the high-affinity *myo*-inositol transport system, with no change in the K_m. This increase was blocked by actinomycin D or cycloheximide. Examination of postinduction normalization showed that returning hyperosmotic-treated cells to isotonic medium caused a rapid reversion of SMIT mRNA levels, followed by a return of *myo*-inositol accumulation to basal values. However, the addition of cycloheximide or actinomycin D partially to totally prevented the reversal in SMIT mRNA levels and activity.

Conclusions. These results suggest that RNA and protein synthesis is required for the hyperosmotic induction of SMIT mRNA levels and *myo*-inositol accumulation by TALH cells. Furthermore, normalization of SMIT mRNA levels and *myo*-inositol accumulation following hyperosmotic induction requires RNA transcription and protein synthesis.

Myo-inositol has at least two important functions in mammalian cells. First, it has an integral role in signal transduction pathways by virtue of its incorporation into phosphoinositides and its subsequent release as secondary messengers on activation of a phosphoinositide-specific phospholipase C or phosphatidylinositol 3-kinase [1–3]. Second, *myo*-inositol is an important osmolyte that protects cells exposed to hyperosmotic stress [4]. This protective role is shared with other osmolytes such as sorbitol, betaine, taurine, and glycerophosphorylcholine; however, these osmolytes may differ in their role as osmotic regulators because of their specific tissue localization and mechanisms responsible for their accumulation/metabolism [5, 6].

In most mammalian cells, the intracellular concentration of *myo*-inositol is maintained at levels many times higher than circulating concentrations [7, 8]. This gradient is regulated and maintained by a Na^+/*myo*-inositol cotransporter (SMIT) that is widely expressed in mammalian cells and by an efflux mechanism, which is poorly understood [9, 10]. In mammalian cells, hyperosmolarity is the most potent means to increase the activity of the SMIT and thus *myo*-inositol accumulation [11–13]. The hyperosmolarity-induced increase in *myo*-inositol transport is dependent on increased transcription of the SMIT gene [12]. Osmotic regulation of the organic osmolytes is physiologically important to renal cells; however, we and others have shown that hyperosmolarity also regulates SMIT activity and mRNA levels in endothelial, neural, and glial cells [11, 13–16]. In addition, by *in situ* hybridization, we have previously localized SMIT mRNA levels in the kidney and demonstrated that the medullary thick ascending limb of Henle's (TALH) loop was an important structure for the concentration of SMIT mRNA [5]. Therefore, we were interested in investigating the osmotic regulation of SMIT mRNA levels and *myo*-inositol accumulation in cells derived from this region of the kidney. In these studies, we demonstrate that hyperosmolarity regulates SMIT mRNA and *myo*-inositol accumulation in renal cells derived from the rabbit outer medullar TALH. Moreover, we show that the reversion of *myo*-inositol accumulation and SMIT mRNA levels, once the hyperosmotic stimulus has been removed, is inhibited by actinomycin D and cycloheximide,

Key words: hyperosmolarity, renal cells, cerebral microvessel endothelial cells, transcription, SMIT.

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suggesting that normalization of SMIT activity and mRNA levels following hyperosmotic induction is regulated in mammalian cells.

METHODS

Materials

The chemicals were from Sigma (St. Louis, MO, USA) unless otherwise noted. Ethanol, chloroform, isoamyl alcohol, Corning 75 cm^2 flasks, and Falcon 6-well plates were from Fisher Scientific (Fair Lawn, NJ, USA). Phenol was from Bethesda Research Laboratories (Gaithersburg, MD, USA). Ethidium bromide and proteinase K were from Boehringher-Mannheim (Indianapolis, IN, USA). Sodium dodecyl sulfate was from BDH (Poole, England, UK). Pyridine, trimethylchlorosilane, and hexamethyldisilazane were from Pierce (Rockford, IL, USA). Acrylamide, bis-acrylamide, dextran sulfate, and N,N,N′,N′-tetramethylethylenediamine were from Biorad (Hercules, CA, USA) or Sigma. Transcription buffer, dithiothreitol, RNasin, adenosine 5′-triphosphate (ATP), cytidine 5′-triphosphate (CTP), uridine 5′-triphosphate (UTP), guanosine 5′-triphosphate (GTP), T7 and SP6 RNA polymerase, and deoxyribonuclease were from Promega (Madison, WI, USA). *Myo*-[2–^{3}H]inositol and [^{32}P]UTP were from Amersham (Arlington Heights, IL, USA). Safety-Solve, cesium chloride, and scintillation vials were from RPI (Mount Prospect, IL, USA). A cDNA probe for the β-actin gene of the rat and 18S rRNA was obtained from Ambion (Austin, TX, USA). Media were obtained from the Diabetes Endocrinology Research Center, University of Iowa (Iowa City, IA, USA).

Cell culture

Murine MB114 cerebral microvessel endothelial (CME) cells were obtained from Dr. Steven Moore, University of Iowa. The cells were grown in M199 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and basal media Eagle amino acid and vitamin solutions [11]. Bovine aortic endothelial cells originated from freshly slaughtered steers and were grown in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 294 μg/ml glutamine [11]. Murine cortical collecting duct and rat inner medullar collecting duct cells were kindly provided by Dr. John Stokes, University of Iowa, and were grown in Dulbecco's minimal Eagle's medium/F12 medium supplemented with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 294 μg/ml glutamine [11]. Thick ascending limbs of Henle (TALH) cells were a kind gift from Drs. Kinne and MacDonald at the Max-Planck-Institut for Molekulare Physiologie (Dortmund, Germany) and the University of Paisley (Glasgow, Scotland, UK), respectively. The cells were derived from the outer medulla of rabbit kidney and were transfected with a plasmid pSV2-neoDNA (which contains the bacterial gene *neo*, which confers resistance to the toxic antibiotic G418) plus DNA from the early region of SV40 [17]. The cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 294 μg/ml glutamine, and Geneticin (0.2 mg/ml). All cells were propagated in Corning 75 cm^2 flasks in an incubator maintained at 37°C with 5% CO_2 in humidified air. Cells were passed weekly at a dilution ranging from 1:10 to 1:20 and were fed three times per week by replacing the medium. For *myo*-inositol accumulation studies, the cells were seeded onto Falcon six-well cluster plates, and assays were conducted in triplicate when the cells reached confluence. For the mRNA studies, the cells were seeded in Corning 75 cm^2 flasks.

Myo-inositol accumulation and cellular *myo*-inositol determination

For *myo*-inositol accumulation determination, cells were incubated in medium for 3 to 24 hours in the absence or presence of 50 to 300 mM raffinose to induce hyperosmolarity [11]. Afterward, the cells were washed with osmotically matched serum-free M199 medium and were then incubated for up to 60 minutes in 2 ml of this medium containing 0.5% bovine serum albumin and *myo*-[2-^{3}H]inositol. The *myo*-inositol concentration of the serum-free medium was 11.4 μM. After the incubation, cells were quickly washed two times with ice-cold 10 mM HEPES buffer, pH 7.4, containing 128 mM NaCl, 5.2 mM KCl, 2.1 mM $CaCl_2$, 2.9 mM $MgSO_4$, and 5 mM glucose and were collected by scraping the cells in 1.5 ml water. The cell suspension was sonicated for 5 seconds, and samples were taken to determine protein content and *myo*-inositol accumulation. *Myo*-inositol accumulation was determined by taking duplicate aliquots of the cell suspension and measuring the radioactivity present using a Beckman LS8100 liquid scintillation counter. Protein content was determined in duplicate aliquots of the cell suspension using a modification of the Lowry method [18]. *Myo*-inositol accumulation is reported as nmol per mg protein. For many of these studies, *myo*-inositol accumulation was determined following a one-hour incubation. In these studies, "*myo*-inositol accumulation" represents the cellular accumulation of radioactivity derived from *myo*[2-^{3}H]-inositol in the medium during the incubation and does not represent the initial rate of *myo*-inositol transport by the cells. It also does not account for any *myo*[2-^{3}H]-inositol taken up by the cells and then secreted during the one-hour incubation period. For the reversion studies, cells were exposed to medium containing 150 mM raffinose (490 mOsm) for 24 hours then were washed with isotonic medium (~300 mOsm) and resuspended in isotonic me-

dium or this medium containing 5 μM actinomycin D or 25 μM cycloheximide for 6 to 24 hours. Afterwards, *myo*-inositol accumulation was determined as described above. For these studies, *myo*-inositol accumulation was also determined in cells maintained in isotonic medium or exposed for 24 hours to hypertonic medium. Cell viability was verified by demonstrating exclusion of trypan blue and the continued adherence of the cells to the culture plate. In addition, ^{51}Cr release studies were conducted to assure that changes in hyperosmolarity did not reduce cellular viability. ^{51}Cr release studies were conducted according to the method of Thorne et al [19]. For these studies, cells were prelabeled with ^{51}Cr for two hours, were washed with normal medium, and were then exposed to either isotonic medium, hypertonic medium, or isotonic medium containing 1% Triton X-100 for one hour. Then the medium was examined for radioactivity. Results from this study with each of the cell lines indicated that exposing the cells for one hour to hyperosmotic medium had absolutely no effect on ^{51}Cr release from the cells compared with cells exposed to isotonic medium (data not shown).

To determine the effect of sodium on *myo*-inositol uptake by TALH cells, cells were incubated in HEPES buffer containing 10 μM *myo*-[2-^{3}H]inositol for 10 minutes. For Na^+-independent *myo*-inositol uptake determination, the cells were washed and incubated in this buffer containing choline chloride in place of NaCl. Kinetic parameters for high-affinity *myo*-inositol transport were determined by incubating cells for five minutes in HEPES buffer with or without NaCl and containing 5 to 250 μM *myo*-[2-^{3}H]inositol as previously described [11]. Uptake of *myo*-inositol occurring in the absence of NaCl was subtracted from uptake in the presence of NaCl before determining the apparent (indicated by the apostrophe) K'_m and V'_{max} for high-affinity *myo*-inositol transport [11].

To determine the effect of hyperosmolarity on the intracellular *myo*-inositol and sorbitol content, TALH cells were grown in 25 cm^2 flasks to confluence and were then incubated in serum-free medium in the absence or presence of 150 mM raffinose for 24 hours. Afterward, the cells were washed with glucose-free HEPES buffer, collected in water, and sonicated. Aliquots of the cell suspension were taken for protein determination and derivatization as described previously [11]. The derivatized samples were then chromatographed on a temperature-programmed Hewlett-Packard 5890 gas chromatograph interfaced with a model HP3390A integrator. The initial temperature of 180°C was maintained for two minutes and was then increased at 4°C/minute to a final temperature of 225°C, which was maintained for five minutes. The column consisted of 3% SE-30 on Supelcoport (Supelco, Bellefonte, PA, USA). An authentic standard containing *myo*-inositol and sorbitol was run to verify elution times, and methyl α-D-mannopyranoside was added as an internal standard [11]. The *myo*-inositol and sorbitol content was calculated as nmol/mg of cell protein.

Quantitation of SMIT mRNA levels

Na^+/*myo*-inositol cotransporter mRNA levels in CME, cortical collecting duct, and TALH cells were quantitated using a solution hybridization-RNase protection assay as previously described [11]. Briefly, [^{32}P]-labeled antisense SMIT mRNAs were transcribed using T7 RNA polymerase and a murine SMIT cDNA construct in pGEM-3Zf(+) that had been linearized with *Hind* III. For the inner medullar collecting duct cells, [^{32}P]-labeled antisense SMIT mRNAs were transcribed using SP6 RNA polymerase and a rat SMIT cDNA construct in pGEM-3Zf(+) that had been linearized with *Eco*R1 [5]. Details of the cloning of the murine and rat SMIT cDNAs have been described previously [5, 11]. The murine SMIT cDNA binds to a 185 nucleotide fragment, and the rat SMIT cDNA binds to a 363 nucleotide fragment. For these studies, because of the high degree of homology of the SMIT gene, the [^{32}P]-labeled antisense murine SMIT mRNA probe was hybridized with rabbit RNA to give a protected band similar in size to RNA derived from murine cells. Antisense SMIT mRNAs were incubated at 45°C in 75% formamide-0.4 M NaCl with 20 μg of total RNA. After 16 hours of incubation, the samples were digested with RNases A and T_1. The protected double-stranded hybrids were collected by ethanol precipitation and electrophoresed through an 8% polyacrylamide-8 M urea denaturing gel. To confirm equal loading of the gel, 18S ribosomal RNA or β-actin mRNA was determined simultaneously with the use of a commercially available 18S antisense control template, which binds to an 80-nucleotide fragment from a conserved region of the 18S ribosomal RNA or a β-actin rat antisense control template that binds to a 127 nucleotide fragment of β-actin mRNA. The antisense 18S and β-actin RNAs were generated per the manufacturer's instructions using T7 polymerase. A sufficient quantity of each of the antisense SMIT mRNA, β-actin, and 18S rRNA probes was added to each sample to insure the presence of an excess of labeled antisense RNA [11]. SMIT mRNAs were represented as a single band on the autoradiogram of the gel, with the intensity of the band being proportional to the SMIT mRNA level in the sample. SMIT mRNA levels were quantitated by scanning densitometry of the autoradiogram using a GS 300 transmittance/reflectance scanning densitometer (Hoefer, San Francisco, CA, USA) interfaced with a model HP 3396A integrator and standardized to the intensity of the 18S rRNA or β-actin mRNA band.

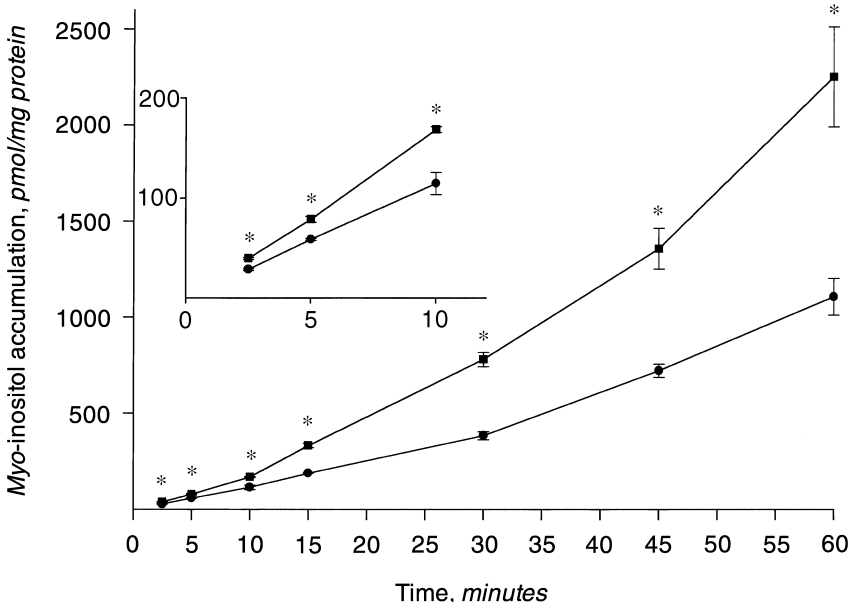


Fig. 1. Effect of hypertonicity on *myo*-inositol accumulation by TALH cells. Cells were incubated for 16 hours in isotonic medium (-●-) or medium containing 150 mM raffinose (-■-); 490 mOsm). Afterwards, *myo*-inositol accumulation was determined as described in the **Methods** section by incubating the cells for 5 to 60 minutes in serum-free medium containing 0.5% bovine serum albumin and *myo*-[2-^{3}H]inositol. Data are expressed as the mean ± SEM for four separate determinations conducted in triplicate. $*P < 0.05$, compared to untreated cells.

Data analysis

Data for *myo*-inositol accumulation are reported as nmol per milligram cell protein or as a percentage of the control. *Myo*-inositol and sorbitol content data are presented as nmol per milligram cell protein. Significance of differences was determined by analysis of variance and Student's *t*-test. For analysis of SMIT mRNA levels, statistical comparisons for significance were performed using the one-tailed multiple comparison procedure of Dunnett.

RESULTS

Effect of hyperosmolarity on *myo*-inositol accumulation and SMIT mRNA levels in TALH cells

Previously, we and others have shown that SMIT mRNA in the outer medulla was predominantly localized to the medullar TALH loop [5, 20]. Therefore, we examined the effect of hyperosmolarity on *myo*-inositol accumulation and SMIT mRNA levels in TALH cells derived from the outer medulla of rabbit kidney [17]. Data in Figure 1 show a time course (5 to 60 minutes) for *myo*-inositol accumulation by TALH cells after a 16-hour incubation in isotonic or hypertonic medium (150 mM raffinose, 490 mOsm). The accumulation of *myo*-inositol was linear in isotonic cells during the initial 30 minutes of the incubation period and then apparently accelerated. Accumulation of *myo*-inositol by hyperosmotic treated cells was linear for the initial 10 minutes of the incubation period (insert, Fig. 1) and was then accelerated. At all time points, *myo*-inositol accumulation was significantly increased following exposure to hyperosmotic medium. The reason for the unusual induction of *myo*-inositol accumulation by TALH cells at the later time points is unknown. TALH cells have a large metabolic capacity for *myo*-inositol, including a large intracellular-free *myo*-inositol pool and vigorous incorporation of *myo*-inositol into phosphoinositides. It is possible that as *myo*-[2-^{3}H]inositol is distributed into these different pools, following its initial uptake, that *myo*-[2-^{3}H]inositol turnover may vary, giving the appearance that *myo*-inositol uptake increases with a longer incubation period. The apparent induction of *myo*-inositol accumulation observed following a 30- to 60-minute incubation may become less dramatic if the incubation period is extended to 6 to 24 hours, which would allow enough time for the *myo*-[2-^{3}H]inositol taken up to equilibrate within the cell. However, this is only one interpretation of these data, and other explanations may be possible.

We have also determined that the accumulation of *myo*-inositol by TALH cells is completely Na^+ dependent. Incubating normal or hyperosmotic-exposed cells in a Na^+-free HEPES buffer containing 10 μM *myo*-[2-^{3}H]inositol for 10 minutes reduced *myo*-inositol accumulation by more than 95% compared with cells incubated in normal HEPES buffer (data not shown). Data in Figures 2 and 3 show the effect of increasing osmolarity (~300 to 590 mOsm) and time of exposure (0.5 to 24 hr) on *myo*-inositol accumulation and SMIT mRNA levels, respectively, by TALH cells. Exposing the cells for 16 hours to 50 to 300 mM raffinose caused an osmolarity-dependent increase in *myo*-inositol accumulation and SMIT mRNA levels. A maximum effect on *myo*-inositol accumulation and SMIT mRNA levels was observed with 200 mM raffinose (490 mOsm) following a 12-hour incubation. Data in Figures 2 and 3 show that hyperos-

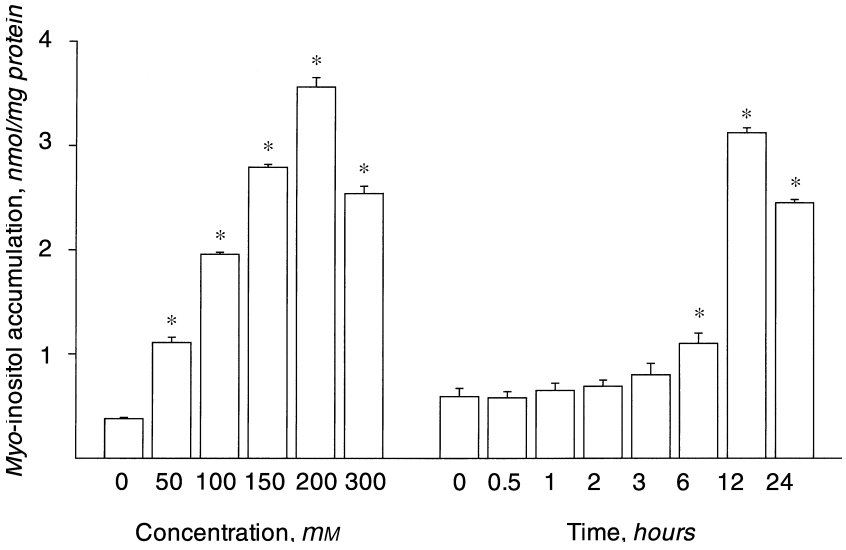
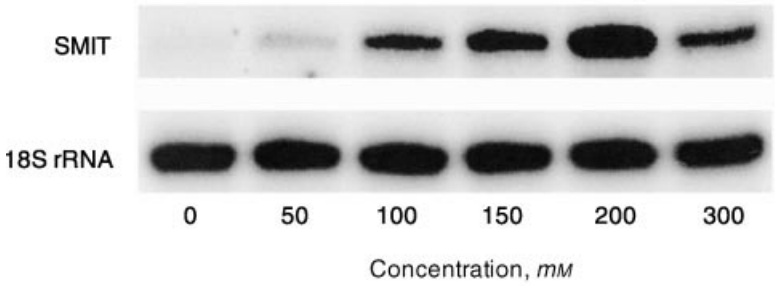


Fig. 2. Concentration and time course analysis of the effect of hyperosmolarity on myo-inositol accumulation by TALH cells. Cells were incubated for 16 hours in medium containing 50–300 mM raffinose (~300–590 mOsm) or in medium containing 200 mM raffinose (490 mOsm) for 0.5 to 24 hours. Afterwards, *myo*-inositol accumulation was determined as described in the **Methods** section by incubating the cells for one hour in serum-free medium containing 0.5% bovine serum albumin and *myo*-[2-^{3}H]inositol. Data are expressed as the mean ± SEM for six separate determinations conducted in triplicate. $*P < 0.05$, compared to untreated cells.



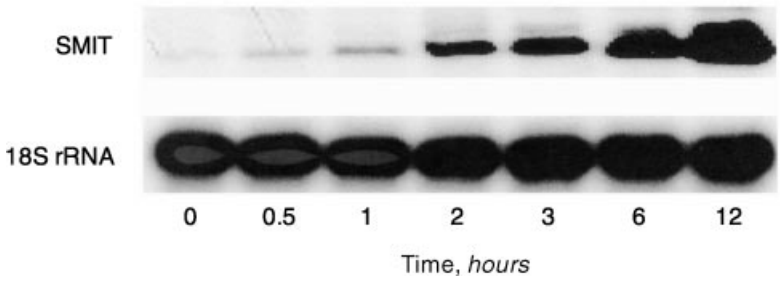


Fig. 3. Representative autoradiograph of the effect of hypertonicity on levels of SMIT mRNA and 18S rRNA in TALH cells. Cells were incubated for 16 hours in medium containing 50 to 300 mM raffinose (~300 to 590 mOsm) or in medium containing 200 mM raffinose (490 mOsm) for 0.5 to 12 hours. Afterwards, RNA was isolated, and SMIT mRNA and 18S rRNA levels determined by RNase protection assay as described in the **Methods** section.

molarity induction of SMIT mRNA levels precedes the increase in *myo*-inositol accumulation by TALH cells. The level of 18S rRNA was unchanged by hypertonicity. Exposing TALH cells for 24 hours to hypertonic medium (200 mM raffinose) caused a significant increase in the intracellular sorbitol and *myo*-inositol content. The sorbitol and *myo*-inositol content of TALH cells after incubation in normal or isotonic medium was 5.0 ± 0.7 and 165.3 ± 18 nmol/mg protein, respectively ($N = 12$). After a 24-hour incubation in hypertonic medium, the sorbitol and *myo*-inositol content was significantly increased to 15.4 ± 1.7 and 336.0 ± 25 nmol/mg protein, respectively ($P < 0.05$).

K'_m and V'_{max} for high-affinity *myo*-inositol transport were calculated to determine whether the hypertonicity-induced increase in *myo*-inositol accumulation by TALH cells was due to a change in *myo*-inositol transporter affinity or to an increase in the number of *myo*-inositol transporters on the plasma membrane. Kinetic analysis of high-affinity transport by TALH cells was conducted,

Table 1. Effect of cycloheximide or actinomycin D on hypertonicity-induced accumulation of *myo*-inositol by TALH cells

	Cycloheximide		Actinomycin D	
	−	+	−	+
Control	0.66 ± 0.02	0.80 ± 0.02	0.30 ± 0.01	0.28 ± 0.01
Hyperosmolarity	3.08 ± 0.33[ab]	0.64 ± 0.04	1.36 ± 0.04[ab]	0.21 ± 0.01

Thick ascending limb of Henle (TALH) cells were preincubated in isotonic medium with or without cycloheximide (25 μM) or actinomycin D (5 μM) for one hour prior to the induction of hyperosmolarity (200 mM raffinose ~ 490 mOsm). The cells were then incubated for 16 hours, and, afterwards, *myo*-inositol accumulation was determined by incubating the cells for one hour in serum-free medium containing 0.5% bovine serum albumin and *myo*-[2-^{3}H]inositol. The final concentration of *myo*-inositol in the medium was 11.4 μM. *myo*-inositol accumulation is expressed as the mean ± SEM for six separate determinations conducted in triplicate.

[a] $P < 0.05$ compared to control

[b] $P < 0.05$ compared to cycloheximide or actinomycin D treated cells, respectively

as previously described, by incubating the cells in buffer with or without Na^+ for five minutes containing 0 to 250 μM *myo*-inositol [11]. The uptake of *myo*-inositol by the cells incubated in buffer without Na^+ was subtracted from the uptake of *myo*-inositol by cells incubated in buffer with Na^+. A five-minute uptake period was selected for these studies because the accumulation of *myo*-[2-^{3}H]inositol after a five-minute incubation was linear for both isotonic and hyperosmotic treated cells (insert, Fig. 1). This time period also avoided the unexplained induction of *myo*-inositol accumulation, which is apparent during later time points (Fig. 1). Background studies for *myo*-inositol uptake by TALH cells demonstrated that after a five-minute incubation period, *myo*-inositol uptake is saturated at an extracellular *myo*-inositol concentration of approximately 1 mM (data not shown). Like many other cell types, TALH cells have a low- and high-affinity *myo*-inositol transport system (data not shown) [21]. The low-affinity transport system is Na^+ independent and is apparent at nonphysiological concentrations of *myo*-inositol, whereas the high-affinity transport system is Na^+ dependent and dominates at physiological concentration of *myo*-inositol. For these studies, cells were incubated in isotonic or hypertonic medium (200 mM raffinose, ~490 mOsm) for 16 hours prior to the analysis of high-affinity *myo*-inositol transport. Results from eight separate studies showed that the K'_m and V'_{max} were 104.3 ± 24.5 μM and 230.2 ± 38.0 pmol/min/mg protein, respectively, for cells incubated in isotonic medium and 103.6 ± 25.3 μM and 711.4 ± 77.2 pmol/min/mg protein ($P < 0.01$), respectively, for cells incubated in hypertonic medium. These results are consistent with the hypertonicity-induced increase in SMIT mRNA levels in TALH cells and suggest that the number of *myo*-inositol transporters on the plasma membrane of TALH cells is increased by exposing the cells to hypertonic medium.

Data in Table 1 show that the hyperosmolarity-induced increase in *myo*-inositol accumulation by TALH cells is significantly inhibited by cycloheximide and actinomycin D. In TALH cells, the hyperosmolarity-induced increase in SMIT mRNA levels was blocked by actinomycin D but not by cycloheximide (Fig. 4). These data suggest that the hyperosmolarity-induced increase in *myo*-inositol transport requires protein synthesis and transcriptional activation of the SMIT gene, whereas the increase in SMIT mRNA levels is dependent on only RNA synthesis [11, 12].

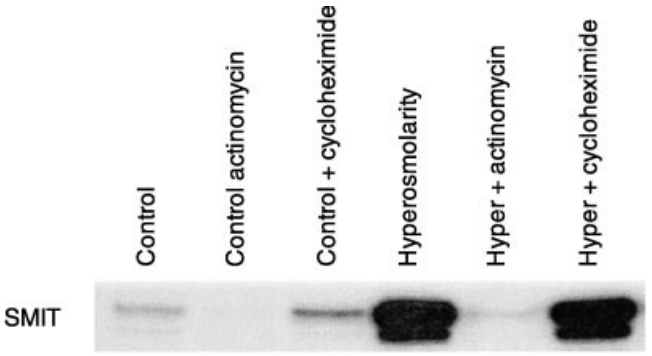


Fig. 4. Representative autoradiograph of the effect of actinomycin D or cycloheximide on the induction of SMIT mRNA levels by hyperosmolarity in TALH cells. Cells were preincubated in serum-free medium containing 5 μM actinomycin D or 25 μM cycloheximide for one hour prior to the induction of hypertonicity. After 12 hours, RNA was isolated, and SMIT mRNA levels determined by RNase protection assay as described in the **Methods** section.

Postinduction normalization of hyperosmolarity-induced *myo*-inositol accumulation and SMIT mRNA levels

Several studies have shown that the reversal of the hyperosmolarity-induced increase in *myo*-inositol accumulation occurs within hours after the removal of the hyperosmotic condition [11, 14–16]. This was confirmed by studies presented in Table 2, which show that within 6 to 12 hours after removal of the hyperosmotic condition, *myo*-inositol accumulation by cultured renal and endothelial cells is significantly reduced compared with the *myo*-inositol accumulation by cells in hyperosmotic conditions and by 48 hours has returned to basal levels. These studies indicate that turnover of the SMIT occurs relatively rapidly; however, little is known about the regulation of this process.

In the following studies, we examined the effect of

Table 2. Post-induction normalization of *myo*-inositol accumulation by cultured renal and endothelial cells following hyperosmotic induction

	Cells *% of control*				
Conditions	TALH	IMCD	CCD	BAE	CME
Hyperosmolarity	1016 ± 15[a]	349 ± 58[a]	461 ± 25[a]	863 ± 84[a]	968 ± 25[a]
Reversion					
6 hours	731 ± 20[ab]	371 ± 69[a]	488 ± 7[a]	326 ± 14[ab]	866 ± 57[ab]
12 hours	441 ± 6[ab]	271 ± 55[a]	276 ± 14[ab]	245 ± 12[ab]	298 ± 5[ab]
24 hours	209 ± 3[ab]	262 ± 62[a]	149 ± 8[b]	207 ± 15[b]	95 ± 7[b]
48 hours	104 ± 4[b]	102 ± 11[b]	111 ± 1[b]	117 ± 7[b]	89 ± 3[b]

Cultured cells were exposed to hyperosmotic medium containing 150 mM raffinose for 24 hours. Afterwards, the cells were washed and incubated for 6 to 48 hours in isotonic medium. After these incubations, *myo*-inositol accumulation was determined by incubating the cells for one hour in serum-free medium containing 0.5% bovine serum albumin and *myo*-[2-^{3}H]inositol. The final concentration of *myo*-inositol in the medium was 11.4 μM. *myo*-inositol accumulation (mean ± SEM) is expressed as the percent of control. For these studies, the accumulation of *myo*-inositol by cells maintained in isotonic medium was 0.41 ± 0.01 ($N = 3$), 0.34 ± 0.03 ($N = 6$), 0.11 ± 0.01 ($N = 3$), 0.36 ± 0.03 ($N = 6$), and 0.89 ± 0.06 ($N = 6$) nmol/mg protein for thick ascending limb of Henle (TALH), inner medullar collecting duct (IMCD), cortical collecting duct (CCD), arota endothelial (BAE), and cerebral microvessel endothelial (CME) cells, respectively.

[a] $P < 0.05$, compared to control

[b] $P < 0.05$, compared to hyperosmolarity-induced cells

Table 3. Effect of actinomycin D and cycloheximide on post-induction normalization of *myo*-inositol accumulation by cultured renal and endothelial cells following hyperosmotic induction

	Cells				
Conditions	TALH (14)	IMCD (14)	CCD (9)	BAE (15)	CME (12)
Control	0.70 ± 0.07	0.35 ± 0.03	0.12 ± 0.01	0.36 ± 0.02	1.48 ± 0.17
Hyperosmolarity	2.13 ± 0.18[ab]	0.76 ± 0.08[ab]	0.65 ± 0.07[ab]	2.17 ± 0.40[ab]	5.78 ± 0.53[ab]
Reversion					
Isotonic	1.37 ± 0.13[a]	0.40 ± 0.05	0.28 ± 0.04	0.74 ± 0.08[a]	2.30 ± 0.23
Isotonic + actinomycin D	2.79 ± 0.21[ab]	0.86 ± 0.06[ab]	1.80 ± 0.16[ab]	2.08 ± 0.41[ab]	6.71 ± 0.78[ab]
Isotonic + cycloheximide	2.52 ± 0.13[ab]	1.06 ± 0.05[ab]	1.97 ± 0.24[ab]	1.86 ± 0.24[ab]	3.93 ± 0.30[ab]

Cells designated to be used in the reversion studies were incubated for 24 hours in hyperosmotic medium containing 150 mM raffinose. Afterwards, the cells were washed with isotonic medium, then resuspended in isotonic medium or this medium containing 5 μM actinomycin D or 25 μM cycloheximide for either 16 hours [cortical collecting duct (CCD), inner medullary collecting duct (IMCD), or cerebral microvessel endothelial (CMD) cells] or 24 hours [thick ascending limb of Henle (TALH) or aorta endothelial (BAE) cells]. Control cells were incubated continuously in isotonic medium, and cells exposed to hyperosmotic conditions were incubated in medium containing 150 mM raffinose for 24 hours prior to determination of *myo*-inositol accumulation. After these incubations, *myo*-inositol accumulation was determined by washing the cells with serum-free medium and then incubating the cells for one hour in this medium containing 0.5% bovine serum albumin and *myo*-[2-^{3}H]inositol. The final concentration of *myo*-inositol in the medium was 11.4 μM. *myo*-inositol accumulation (nmol/mg protein) is expressed as the mean ± SEM for the number of determinations indicated in the parentheses.

[a] $P < 0.05$ compared to control

[b] $P < 0.05$ compared to hyperosmolarity-induced cells returned to isotonic medium

cycloheximide and actinomycin D on the reversion of hyperosmolarity-induced SMIT mRNA levels and *myo*-inositol accumulation using cultured renal and endothelial cells. The concentrations of cycloheximide and actinomycin D used in these studies were shown to block protein synthesis and gene transcription, respectively (data not shown). For these studies, the cells were exposed to 150 mM raffinose for 24 hours. Afterwards, the cells were washed and resuspended in isotonic medium or this medium containing 5 μM actinomycin D or 25 μM cycloheximide for 16 to 24 hours, and then the SMIT mRNA levels and *myo*-inositol accumulation were determined. Cells incubated for 24 hours in isotonic medium or in this medium containing 150 mM raffinose were used to determine basal or hyperosmolarity-induced SMIT mRNA levels and *myo*-inositol accumulation. Data in Table 3 show that exposing each of the five different renal or endothelial cell types to hyperosmotic medium for 24 hours caused a significant increase in *myo*-inositol accumulation. Returning cells exposed to hyperosmotic medium to isotonic medium for 16 to 24 hours caused a significant reduction in *myo*-inositol accumulation, which approached basal levels. However, if either actinomycin D (5 μM) or cycloheximide (25 μM) is added to the isotonic medium, *myo*-inositol accumulation remains elevated and is significantly greater than the *myo*-inositol accumulation observed in cells preincubated in hyperosmotic medium and returned to isotonic medium for 16 to 24 hours. Data in Figure 5 show that SMIT mRNA levels were significantly elevated in each of the cell types after a 24-hour exposure to hyperosmotic medium (condition 2), and they returned to near basal levels after returning the hyperosmolarity-induced cells to isotonic medium for six hours (condition 3). Adding 5 μM actinomycin D (condition 4) or 25 μM cycloheximide (condition 5) to the isotonic medium partially to com-

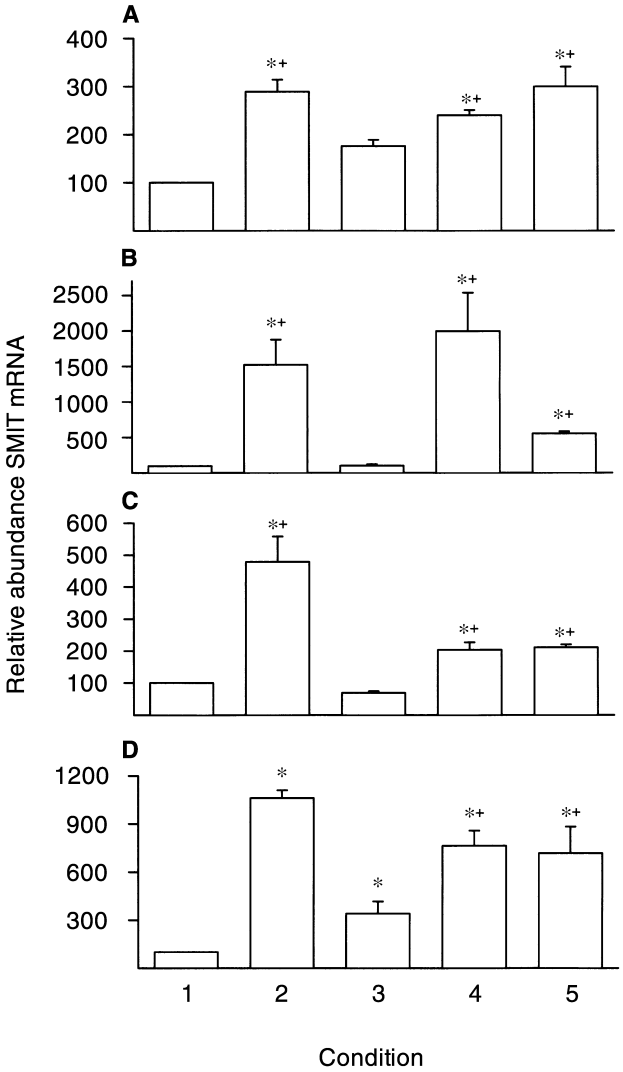


Fig. 5. Effect of actinomycin D or cycloheximide on the post-induction normalization of SMIT mRNA levels in cortical collecting duct (*A*), cerebral microvessel endothelial (*B*), inner medullary collecting duct (*C*) and thick ascending limb of Henle (*D*) cells. Cells were incubated as described in Table 3. After a six hour incubation, RNA was isolated and SMIT mRNA levels determined by RNase protection as described in the **Methods** section. The relative abundance of SMIT mRNA in control cells maintained in isotonic medium was arbitrarily assigned a value of 1. All values were normalized using β-actin mRNA or 18S rRNA levels. $^{*}P < 0.05$, compared to SMIT mRNA levels in control cells. $^{+}P < 0.05$, compared to SMIT mRNA levels in hyperosmolarity-induced cells returned to isotonic medium for 6 hours.

pletely blocked the reversion of SMIT mRNA levels in all four cell types examined. Figure 6 is a representative autoradiograph showing SMIT mRNA levels and either 18S rRNA or β-actin mRNA levels as a control for each of the cell types examined.

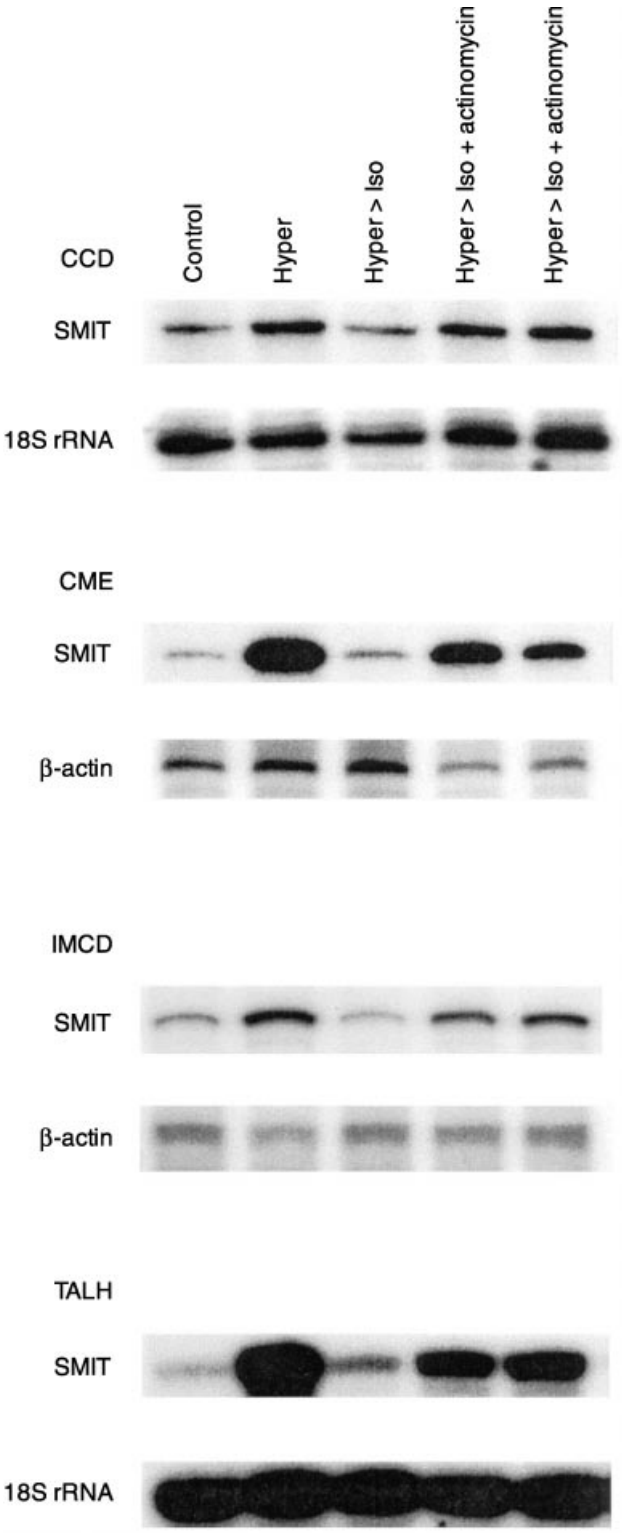


Fig. 6. Representative autoradiograph of the effect of actinomycin D or cycloheximide on the post-induction normalization of SMIT mRNA levels in cortical collecting duct (CCD), cerebral microvessel endothelial (CME), inner medullary collecting duct (IMCD) and thick ascending limb of Henle (TALH) cells.

DISCUSSION

Cells react to increased osmolarity with numerous changes in gene expression. The specific genes affected

differ between species, but the known osmoprotective effects of the gene products are remarkably similar, particularly with regard to cellular accumulation of compatible organic osmolytes [22]. These studies show that cells derived from the outer medullar TALH, like other mammalian cells, increase SMIT mRNA levels and *myo*-inositol accumulation following exposure to hyperosmotic stress [11–13]. Grunewald et al have shown that choline transport by these cells is up-regulated by hyperosmolarity [23]. Similar to the effect of hyperosmotic stress on the choline transport system, the V'_{max} for the high-affinity *myo*-inositol transporter was significantly increased by hyperosmolarity with little change in the K'_m. In addition, our studies showed that sorbitol accumulation by TALH cells is increased following exposure to hyperosmotic medium. Thus, these studies demonstrate that TALH cells, after exposure to hyperosmotic stress, up-regulate transport and synthetic pathways to increase the production and/or uptake of the organic osmolytes, glycerophosphorylcholine, sorbitol, and *myo*-inositol [23]. However, as our studies also show, *myo*-inositol accumulation by TALH cells is several-fold greater than sorbitol accumulation, suggesting that *myo*-inositol may be the predominant osmolyte in these cells. This would agree with previous *in vivo* studies of renal SMIT distribution, which demonstrated that the TALH loop is the major site of localization of SMIT mRNA in the kidney [5, 20].

In other renal cell types, the effect of hyperosmolarity on SMIT gene expression is at the level of gene transcription [12]. Our data are consistent with the hyperosmolarity-induced increase in SMIT gene expression in TALH cells being mediated at the level of gene transcription because actinomycin D prevented the increase in SMIT mRNA levels. Like the genes encoding the betaine transporter and aldose reductase, the SMIT gene has recently been shown to have an osmolarity-responsive promoter that likely contributes to the increase in SMIT mRNA levels and *myo*-inositol accumulation following osmotic stress [24–27]. From these studies we conclude that TALH cells, like other renal cells, are highly responsive to osmotic stress and rapidly initiate pathways leading to the accumulation of organic osmolytes, and that *myo*-inositol may be the primary osmolyte for this region of the kidney.

Reversion of *myo*-inositol accumulation to a value not significantly different than control cells maintained in isotonic conditions following removal of a hyperosmotic stimulus occurs within 16 to 48 hours, as evidenced by studies of several renal cell types, brain glial cells, CME cells, and neuroblastoma cells [11, 14–16]. The studies presented in Table 2 provide additional evidence that reversion of *myo*-inositol accumulation to basal levels occurs within 24 to 48 hours after renal or endothelial cells treated with hypertonic medium are returned to an isotonic medium. We and others have also shown that SMIT mRNA levels revert to normal levels within four to six hours after cells stimulated with hyperosmotic medium are returned to an isotonic medium [11, 16]. This suggests that down-regulation of transporter activity occurs relatively slowly compared with SMIT mRNA levels. Presumably, this rapid reduction in SMIT mRNA levels reflects rapid inhibition of gene transcription in response to a decrease in cell osmolarity and/or a change in mRNA stability. Our studies demonstrate that the reversion of the hyperosmolarity-induced increase in SMIT mRNA levels and *myo*-inositol accumulation to basal levels following the return of cells to isotonic medium is sensitive to RNA and protein synthesis. Actinomycin D and cycloheximide prevented the reversion of *myo*-inositol accumulation to basal levels after cells treated with hyperosmotic medium were returned to isotonic medium. Moreover, the return of SMIT mRNA levels to basal levels under these conditions was partially prevented by actinomycin D and cycloheximide. These data suggest that reversion of SMIT mRNA levels to basal levels is not secondary to the extinction of hyperosmolarity-induced changes in SMIT gene transcription and/or mRNA stability. Rather, reversion appears to be an active and possibly regulated process that is dependent on both RNA and protein synthesis. Certainly, there is a precedent for protein binding to the 3′ untranslated region of mRNAs regulating mRNA stability. For example, tumor necrosis factor-α destabilizes endothelial nitric oxide synthase mRNA by increasing the activity of cytosolic proteins that destabilize endothelial nitric oxide synthase mRNA by binding to its 3′ untranslated region [28]. Interestingly, we have shown that tumor necrosis factor-α causes a decrease in SMIT mRNA levels and transporter activity by an unexplained mechanism in endothelial cells [29]. One possibility for our findings is that the return of cells to isotonic medium induces the transcription of a gene that encodes a protein, which binds to and destabilizes SMIT mRNA. Our data, however, do not rule out the alternative possibility that cells produce a factor after return to isotonic medium that represses expression of the SMIT gene. Further studies will be required to address this issue. Moreover, our studies show that the reversion of *myo*-inositol accumulation to basal levels following the transfer of cells from hyperosmotic to isotonic medium is completely blocked by cycloheximide and actinomycin D. In some cell types, the uptake of *myo*-inositol is even greater in cells transferred from hyperosmotic medium to isotonic medium containing actinomycin D or cycloheximide compared with cells maintained in hyperosmotic medium. The reason for this difference is unknown. These data suggest that SMIT protein turnover is blocked by these conditions and that *myo*-inositol accumulation is maintained or even increased compared with *myo*-inositol accumula-

tion by cells treated with hyperosmotic medium. Whether this effect can be explained solely by a regulation of changes in SMIT mRNA levels or whether changes in SMIT internalization and/or degradation also contribute to the normalization of SMIT activity when cells are returned to isotonic medium from hyperosmotic conditions remains to be determined.

In summary, expression of the SMIT in TALH cells is regulated by hyperosmolarity in a similar manner to other cells. Interestingly, these studies have demonstrated the novel finding that the normalization of SMIT mRNA levels and *myo*-inositol transport after loss of the hyperosmotic stimulus requires both protein and RNA synthesis.

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